

THE MODE OF ACTION OF INSECT REPELLENTS II: ELECTROPHYSIOLOGICAL STUDIES

M. J. REDDY

Department of Entomology
University of Alberta
Edmonton, Alberta

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Apparatus was designed to record sensory nerve impulses coming from the cerci, antennae, and legs of American cockroaches. Recordings were made after stimulation of these sensory zones with an attractant, repellents, and other irritant chemicals. The cercal receptors could not be shown to respond to any chemical stimulation. The leg receptors responded to repellents and irritant chemicals but not to the attractant. The antennal receptors responded to repellents and irritant chemicals plus the attractant. The type of response from both leg and antennal sensory nerves was similar for both repellents and irritant chemicals, when applied either as a liquid or as a vapour. The response of the antennal receptors to attractant vapour was characteristically different.

Recordings were also made of receptor responses from the German cockroach to the repellent MGK R-874. The cercal receptors did not respond. A response to both liquid and vapour was obtained from the leg and antennal receptors. A weak response to repellent liquid only was obtained from the palp receptors.

Possible mechanisms of repellent action are discussed.

Although behavioural studies remain the most important methods in evaluating insect repellents, such experiments are subject to the physiological state and individual variation of the insects used. Statistical comparisons between the presence or absence of a single given stimulus can be made when batches of insects are tested under identical background conditions. In practice however, it is not possible to exclude all other stimuli. This can result in complicated interactions, since any behavioural response other than the simple reflex arc is the outcome of a complex processing in the brain. The study of electrophysiological recordings taken from the sensory nerves is more objective. By comparing nerve responses after chemical stimulation of the sense organs to the resting discharge rate obtained in the absence of known stimuli one is observing sensory inputs on the way to the brain. Any obvious and consistent differences observed in these nerve responses can be interpreted to give direct evidence of the location of the receptors involved with insect repellents. By comparing the nerve responses to other stimuli, such as mechanical ones, with the responses to a combination of repellent and mechanical stimulation, one can also show whether repellents interfere with the reception of other stimuli at the receptor sites or whether they act independently at these sites. Should the various stimuli act independently at the receptor sites, any effect by the repellent on the normal responses to other stimuli is the result of interaction in the brain.

No satisfactory method has been devised for recording the electrical responses of single sense organs being stimulated by vapours. Some success was achieved by Morita and Yamashita (1961) but the method used is inconsistent in results when compared with Hodgson's (1957) method for recording the electrical responses from labial chemosensory hairs of the blowfly to water soluble chemical stimuli. Antennal preparations have been used by a great number of authors to characterize odour responses (Roys, 1954; Smith and Roys, 1955; Boistel, Lecompte and Coraboeuf, 1956; Schneider, 1957a; Roessler, 1961; Schneider and Boeckh, 1962; Boeckh, 1962; Schneider, Lacher and Kaissling, 1964; Lacher, 1964; Schneider, Block, Boeckh and Priesner, 1967). Large probes placed in the antennae of insects to record the activity from the antennal nerve do not record potential spikes of the frequency

that would be expected from a single nerve fibre, but record overall potential changes resulting from the stimulation of a large number of sensory neurons. Such a slow overall change in electrical potential has been called an electroantennogram (Schneider, 1957a). In the present investigation this preparation has been used to compare the antennal response of cockroaches to various attractants and repellents, in the vapour phase and in the liquid phase. A similar preparation has been used to study the effect of these chemicals on the cockroach leg receptors and the effects compared with those obtained from the antennae.

MATERIALS AND METHODS

Electrodes. — The electrodes used in most experiments were tungsten wire. The wire was sharpened to a point by dipping in molten sodium nitrite and then polished electrolytically after a method described by Hubel (1957). The tungsten needles were insulated to the tip by dipping in Insulex, a vinyl lacquer (Donaldson, 1958). The impedance of tungsten electrodes is quite high, depending on the size of the uninsulated tip, but decreases as the frequency of an applied varying potential increases (Donaldson, 1958). This impedance variation in tungsten and other polarizable electrodes makes them unsuitable for the measurement of static potentials such as the cell membrane resting potential, but they are quite suitable for comparative studies of nerve action potentials, providing two identical electrodes are used. They have the advantage of being mechanically robust compared with the fragile glass micro-pipettes of the non-polarizable silver-silver chloride electrodes. Such standard silver-silver chloride electrodes (Donaldson, 1958) were also used in a few preparations to check the results obtained with the tungsten electrodes. These were quite comparable; both tungsten and glass electrodes had an impedance of one to two megohms.

The preamplifier. — Shielded leads from the electrodes were connected to the input (push-pull) stage of a battery operated Grass p-8 d-c preamplifier. The intrinsic noise level of this model is rated at 20 microvolts at the maximum amplification of 2000, and is a little greater than this in practice. This means that any detectable spike must be greater than 30 microvolts. High sensitivity of this order is necessary for a number of reasons. Although the action potential across the membrane of a single nerve fibre is of the order of 100 millivolts (Hodgkin, 1951), the full potential can only be detected by electrodes placed directly on the membrane, and the recorded potential drops rapidly as the electrode distance from the nerve fibre increases. This drop in potential is most often the case with recordings from whole nerves or bundles of fibres, as in these experiments. Types of response are identified by frequency not by amplitude.

The oscilloscope. — The output leads from the preamplifier were connected to the d-c difference input terminals of a Tektronix dual-beam oscilloscope. With these connections, the oscilloscope records only the difference in absolute potential between the two input leads and potential fluctuations affecting both probes are not registered.

Permanent recordings were made with a Polaroid Land camera fixed on the bezel mount flange of the screen. High contrast positive transparency film was used. The single shot nature of the camera limited the number of recordings that could be made. Most of the results are based on written notes taken during visual observations of the screen.

Methods of stimulation

Bursts of electrical activity in the ventral nerve cord of a cockroach are produced when the cerci are stimulated mechanically with a puff of clean air from an eye dropper. A similar eye dropper could be filled with a repellent or attractant vapour and any of the preparations could be subjected to a puff or treated air.

Vapour stimulation without the accompanying puff was achieved by merely holding a glass rod which had been dipped in the liquid repellent or attractant, close to the preparation.

Repellents were applied in liquid form with a squirrel hair paint brush.

The most common method of delivering a vapour stimulus to electrophysiological preparations is by adding the chemical stimulus to a continuous stream of vapour blown over the preparation. This tends to produce mechanical vibrations of the preparation which are transduced into electrical pulses (Schneider, 1957b). A more gentle method of delivering a vapour stimulus was devised. The leg or antennal preparation was set up on a cork stopper in a glass vial so that the leg or antenna protruded through a hole in the stopper into the lumen of the vial. Two pieces of glass tubing were welded into the bottom of the vial and rubber hose attached to these. A slow water flow was maintained through these tubes across the bottom of the vial. A hypodermic syringe could be filled with saturated chemical vapour, and a bubble of known volume injected into the rubber tubing. The bubble would travel slowly along the tubing and pop up into the vial, exposing the preparation to whatever vapour the bubble contained without causing any violent mechanical artifacts. This preparation was only good for vapours insoluble in water, and for single stimulations or combined effects, since there was no way of removing the vapour once it had been delivered.

Repellents used were MGK R-874 (2-hydroxyethyl-n-octyl sulphide), dimethyl phthalate and diethyl toluamide; other chemicals used included benzene, toluene, and ether; the attractant used was banana vapour. Ripe bananas produced a vapour extremely attractive to cockroaches, although the active ingredients have not been determined (Dethier, 1947).

More sophisticated apparatus was available for the *Blattella* work than that used with *Periplaneta*, but the methods and techniques were basically the same. Silver-silver chloride electrodes were used, the reference electrode being placed generally in the body of the cockroach and the recording electrode placed by micromanipulator on the desired nerve via a fluid-filled microcapillary. The signals went through a Medistor A-35 electrometer amplifier (single-sided input) and a Tektronix Type 122 amplifier to a Tektronix 502 oscilloscope. Permanent records were taken with a Grass C4 camera, which inverts the traces (positive is down in Fig. 9-15). The apparatus was capable of making continuous recordings of greater sensitivity than the apparatus described in the previous section. The nerve activity recorded ranged from 8–1000 Hz, in contrast to the slow d-c shifts recorded with *Periplaneta*.

RESULTS

American cockroaches *Periplaneta americana* (L.) were mainly used for this work because their size made them convenient for operation. They were readily available from laboratory cultures, and their nervous anatomy is well known.

Probes were placed with the aid of two Leitz micromanipulator units mounted on a cast steel base. The operation was observed through a Zeiss binocular stereomicroscope. Four basic insect preparations were used:

Cercal preparation (American cockroach). — Decapitated cockroaches were dissected from the dorsal side, revealing the ventral nerve cord. Probes were placed under the ventral nerve cord about 1 mm apart and the cord lifted slightly off the underlying tissue. The abdominal cavity was then filled with mineral oil, which prevented desiccation as well as stimulation of the cord itself due to the presence of repellent vapour (Roys, 1954).

Mechanical stimulation of cockroach cerci either by a puff of air or by touching with a needle produces easily recorded electrical activity in the ventral nerve cord. Spikes of various amplitudes may be present and in general it can be said that spikes of different amplitudes represent recordings from different nerve fibres. Since the action potential of

all nerve tissue is about 100 millivolts (Hodgkin, 1951) two main factors are responsible for different amplitudes being recorded. These are: the distance of the probes from the various nerve fibres, since recorded potential drops rapidly as the distance of the probe from the neuron membrane increases; the size of the neuron in question, since although the action potential of all neurons is similar the current is not, and a large current from a large fibre records as a higher potential than a small current from a small fibre. The frequency of the recorded spikes from the cercal preparation depends on the intensity of the stimulation, the greater the stimulation the greater the frequency (Roeder, 1953). Action potentials recorded from the abdominal cord in a cercal preparation to a burst of stimulation such as a puff of air are bursts of high frequency spikes which adapt slowly approaching the normal resting activity of the nerve. No difference in the spike amplitude or frequency of the recordings was observed between a cercal preparation stimulated with clean air and the same preparation stimulated by a puff of air containing any chemical whatsoever, repellent or attractant. Furthermore, the mechanical response was not affected by painting the cerci with liquid repellent. It should be noted here again that the ventral nerve cord was covered with mineral oil to prevent it being directly affected by chemical vapours. Refined mineral oil itself does not appear to affect the preparation in any way other than to increase its longevity by preventing desiccation.

Complete antennal preparation (American cockroach). — The head of the cockroach was removed from the body and dissected in the eye region to reveal the antennal nerve. Two probes were placed on the nerve and the exposed preparation was covered with mineral oil. Since, in this preparation, the brain was still intact and connected to the antennal nerve, the recordings were complicated by signals going from the brain through the motor neurons.

Detached antennal preparation (American cockroach). — A cockroach antenna was cut off near the base and a reference electrode inserted well into the antenna lumen. Fluid soon congealed in the space between the electrode and the antennal walls, preventing desiccation of the interior of the antenna. A very fine recording electrode was inserted at a joint in the antenna, usually between segments 5 and 6 (Roys, 1954). Beyond the first antennal segments, there are no muscles in a cockroach antenna, and any signals received from this type of preparation are of sensory origin.

Antennal preparations where the antennal nerve was still attached to the brain produced spikes which could be associated with the antennal muscles (Fig. 1). These spikes were of a frequency range normally associated with nerve — muscle preparations, about 100 spikes per second. These unwanted signals disappeared in preparations where the antenna was removed from the head, and the only action potentials recorded from such preparations in still clean air, were from weak mechanical vibrations (Fig. 2). These mechanovibrations just showed above the 20 microvolt limit of amplification of the apparatus. Neither the muscle spikes nor the response to mechano-vibrations could be confused with the 40 times slower frequency changes of the electroantennograms. Slow potential changes of the electroantennogram are thought to be the summed potentials coming from the many receptors on the antenna. The cockroach antenna responded quite violently to stimulation by banana vapour (Fig. 3). The response to repellent vapours was much less marked (Fig. 4 right) and closely resembled in amplitude the continuous stimulation produced when the antenna was painted with liquid repellent (Fig. 5). Breaks appeared in the response to continuous repellent application (Fig. 5). Such breaks were also noted by Roys (1954). After stimulation with repellent vapour the response of the antenna to stimulation by attractant vapour was considerably reduced for several minutes (Fig. 4). Full recovery was effected after about 20 to 30 minutes. Benzene, toluene, and ether vapours acted very similarly to the vapours of repellents such as MGK R-874, diethyl toluamide, and dimethyl phthalate (Fig. 6).

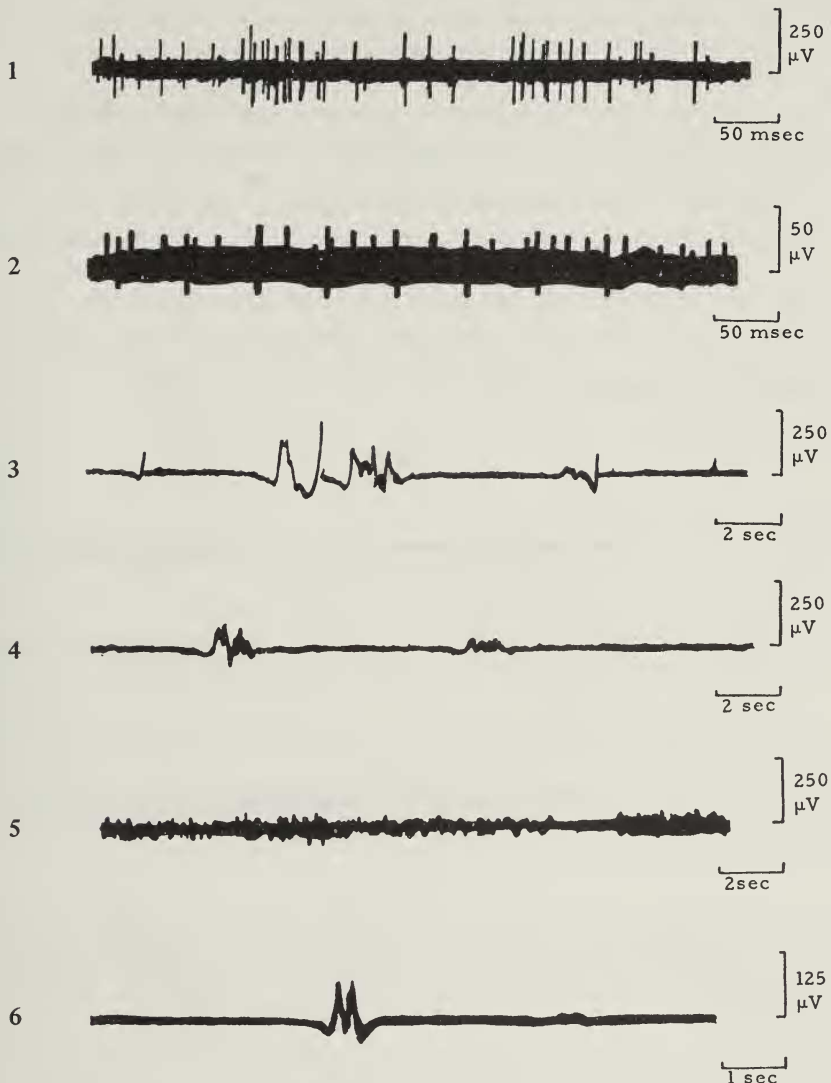


Fig. 1-6. *Periplaneta americana*. 1. Complete antennal preparation. Nerve-muscle potentials whose presence makes this type of preparation unsuitable for investigations into chemosensory responses. Note the frequency of the responses from the time base. 2-6. Detached antennal preparations. 2. Action potentials recorded from an isolated antenna in still clean air. These weak potentials were the only noted steady state responses from the preparation. The baseline of about $20\mu\text{V}$ shows the limit of amplification of the recording apparatus. 3. Electroantennogram from an antenna stimulated with an attractant, banana vapour. Stimulus applied by holding a glass rod which had been dipped in crushed banana, near the preparation. Note the time base; this is a very slow-changing potential. 4. Responses to stimulation with banana vapour (left) and repellent vapour (right), 5 minutes after exposing the preparation to repellent vapour for 1 minute. The repellent was MGK R-874, and the stimuli were applied with glass rods. The decreased amplitude of the attractant response (cf. Fig. 3) shows the very slow recovery of the preparation from the effects of repellents. 5. Response to liquid repellent MGK R-874 painted on the antenna. The amplitude of the response is similar to that obtained from repellent vapour (Fig. 4). The breaks seen in the response to continuous stimulation were noted by Roys (1954). 6. Response to benzene vapour delivered by the bubble apparatus. Allowing for the greater scale of this recording compared with that of Fig. 3, 4, and 5, this response is similar to that produced by insect repellents.

Leg preparation (American cockroach). — The decapitated cockroach was dissected in the coxal region of the foreleg. The main nerve leading from the leg (ganglionic nerve No. 5) was severed from the ganglion and probes placed on it. The exposed region was covered with mineral oil to prevent desiccation.

The cockroach leg did not respond to the attractant vapour at all. The leg did respond to repellent vapours in very much the same way as the antenna (Fig. 7). Repellent liquids painted on the leg produced the same sort of response as vapours except that the duration of response was much greater. I could detect no difference in the response of the legs to stimulation by liquid repellents from the response of the antennae (Fig. 8). The presence of repellent liquid on the leg caused stimulation for a great length of time, breaks in the stimulation gradually becoming longer and longer. However, activity was still noted even after an hour and a half (Fig. 8). Benzene, toluene, and ether all acted similarly to repellents when applied to the leg in either liquid or vapour form although the response, particularly to ether, was slightly more pronounced.

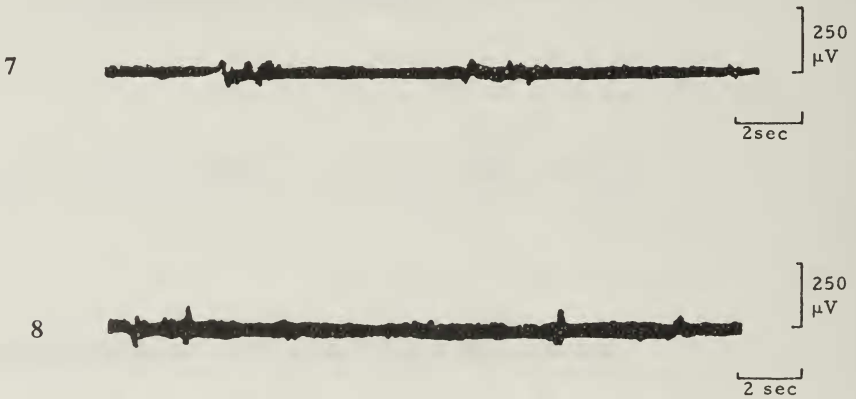


Fig. 7–8. *Periplaneta americana*, leg preparations. 7. Response to repellent vapour, dimethyl phthalate. Two separate stimulations from a treated glass rod. The leg is not as sensitive as the antenna, but the form of the response is very similar. 8. Recording taken 90 minutes after painting the leg with liquid DMP. The breaks between the bursts of activity are much longer than they were just after the onset of stimulation, cf. antennal preparation, Fig. 5.

Although most of the experiments were done with the American cockroach, some tests were carried out with the German cockroach *Blattella germanica* (L.) so that the results could be compared with previous behavioural findings (Reddy, 1970). Adult male German cockroaches were used for all the following experiments. The insects were between 3 and 10 days old and had been reared at 23 C. Each specimen was anaesthetized with carbon dioxide, attached to a wax coated slide and allowed to recover for 30 minutes. The probes were placed in the cockroach and the recordings made in the dark. The repellent stimuli, both vapour and liquid were applied by means of a fine glass rod which had been dipped in MGK R-874. A flashlight was used during stimulus application, since the bench light caused 60 cycle interference. The light itself did not appear to affect the preparation.

Cercal preparation (German cockroach). — The recording electrode was placed on one of the two cercal nerves posterior to the last abdominal ganglion, and the exposed preparation covered in mineral oil. Although this preparation responded well to mechanical stimuli, no response was obtained when a glass rod dipped in MGK R-874 was held near the cercus. When liquid MGK R-874 was applied to the cercus with a glass rod there was a short initial response to the mechanical stimulation, but no further response to the repellent itself (Fig. 9).

Antennal preparation (German cockroach). — The recording electrode was placed in the antenna in the region of the sixth antennal segment. When a glass rod dipped in MGK R-874 was held near the antenna, a sharp burst of electrical activity lasting about 1 second was recorded (Fig. 10). When the antenna was painted with liquid MGK R-874, activity of similar amplitude and duration was noted (Fig. 11). This was followed by a resting period of about 2 seconds, another burst of activity, a resting period of 3 seconds, more activity and so on, with the periods of inactivity getting longer. Similar patterns of activity were observed when the preparation was subjected to continuous stimulation by MGK R-874 vapour. This pattern was also noted by Roys (1954). The antennal responses to MGK R-874 were obtained in 9 out of 10 attempts.

Leg preparation (German cockroach). — With the recording electrode placed in the tibial region of an isolated cockroach foreleg, responses could only be obtained in 5 out of 10 attempts, probably due to the greater difficulty in placing the probe on the nerve. The response of the leg to MGK R-874 vapour was similar to that of the antennae, but of shorter duration (about $\frac{1}{2}$ second, Fig. 12). The response of the leg to MGK R-874 liquid was $\frac{1}{2}$ second bursts of activity with intermittent resting periods similar to those noted for the antennae (Fig. 13).

Palp preparation (German cockroach). — The recording electrode was placed in the first segment of a labial palp, and the rest of the mouthparts sealed with wax to prevent undue mechanical activity. No response to MGK R-874 vapour could be obtained (Fig. 14). In four out of six attempts the palps responded to MGK R-874 liquid with irregular bursts of activity and resting periods (Fig. 15). The resting periods were shorter than those observed in the legs and antennae.

The electrophysiological experiments with *Blattella* confirmed the general conclusions reached with *Periplaneta*. Both the legs and antennae can respond to both the vapour and liquid phases of repellent. The type of nervous response was similar for both liquid and vapour with the legs and antennae of *Blattella*. The bursts of activity produced by the legs were of shorter duration than those produced by the antennae. The situation with the palps is not clear; I could detect a response to liquid MGK R-874 but not to vapour. It is possible that the small number of chemosensory organs present on the palps are contact chemoreceptors in the sense that they respond only to very high concentrations of chemical stimuli.

DISCUSSION

My experiments with the American cockroach indicate that insects respond similarly to both repellents and irritant chemicals such as benzene, toluene, and ether. This is not surprising; benzene for instance, is a known insect repellent, but it has no commercial application since it is so volatile.

Roys (1954) noted that the exposed ventral nerve cord of insects can be stimulated by irritant chemicals. Even if the response to an irritant chemical stimulus is a fundamental

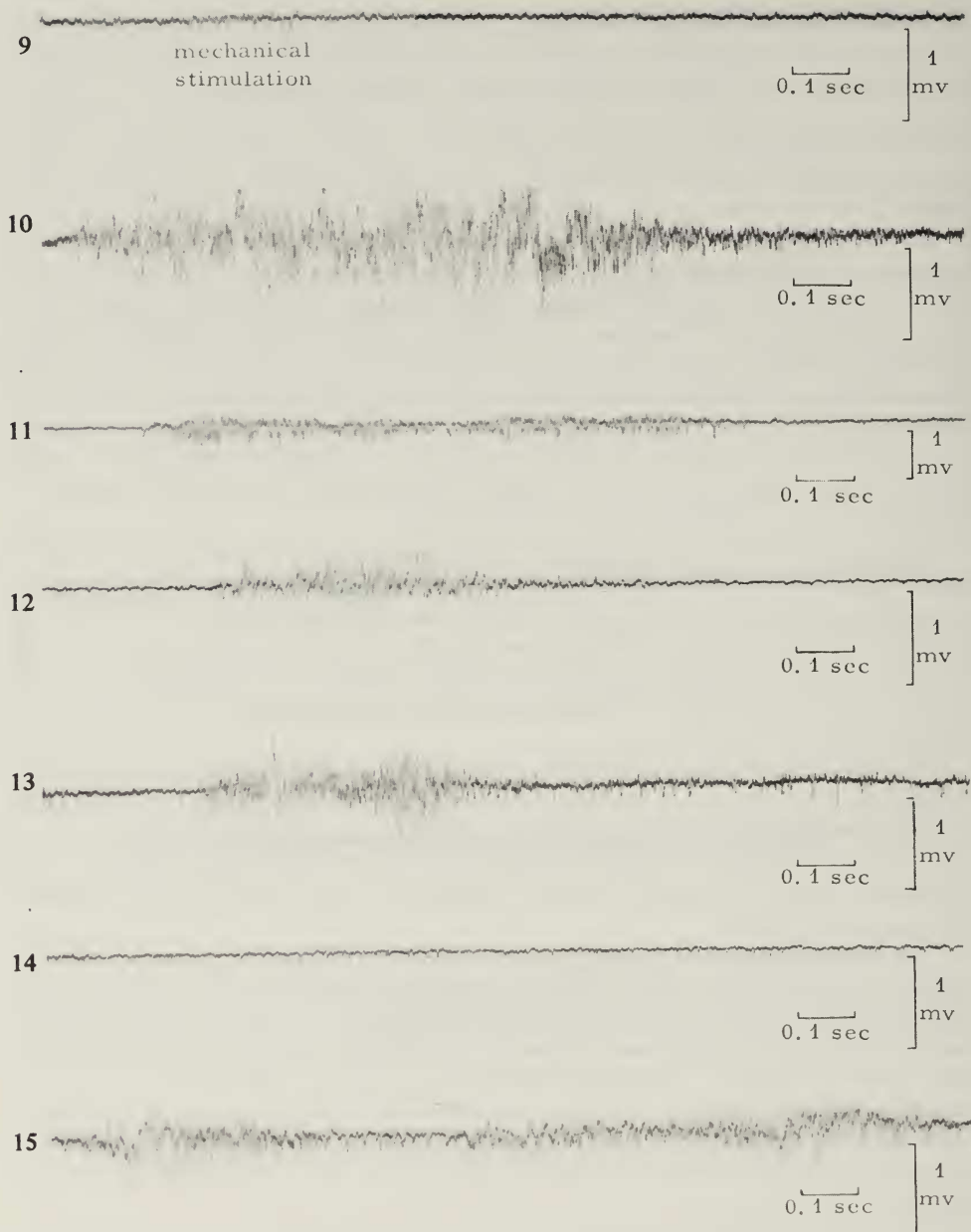


Fig. 9-15. *Blattella germanica*. 9. Recording from the left cercal nerve. After the slight initial response to mechanical stimulation, no activity was produced by MGK R-874 liquid. 10. Response of the right antennal nerve to stimulation by MGK R-874 vapour. 11. Response of the right antennal nerve to stimulation with MGK R-874 liquid. The vertical recording scale is one half that of Fig. 10, which makes both these responses of the same order of amplitude. This response was followed by alternating resting periods and further activity. 12. Response from the right foreleg after stimulation with MGK R-874 vapour. 13. Response from the right foreleg after stimulation with MGK R-874 liquid. This response was followed by alternating resting periods and further activity. 14. Recording from the left labial palp. Lack of response to stimulation by MGK R-874 vapour. 15. Recording from the left labial palp. Response to MGK R-874 liquid. This response was followed by irregular resting periods and activity.

property of all nervous tissue, that tissue must be exposed to the chemical for any response to be noted. The sensory nerves of insect mechanoreceptors are not exposed to the surface, but are protected by the insect cuticle which is a good barrier to most chemicals (Ebeling, 1964). This may explain why I could demonstrate no effect by repellents on mechanoreception in the American cockroach. For chemoreception to be possible, chemicals must have access to sensory tissue, and insect chemoreceptors have pores or openings which make this possible, (Dethier, 1955; Slifer and Sekhon, 1962). Indeed, the chemoreceptors and possibly the hygroreceptors seem to be the only locations on insects where nervous tissue is exposed to stimulation by chemicals, and for this reason are the areas where insect repellents act.

In a previous paper (Reddy, 1970) behavioural evidence was given for locating the main sensory areas on the German cockroach to the repellent MGK R-874 on the antennae, with sensory areas of secondary importance on the legs. The vapour phase of the repellent was more effective than the liquid phase. Combining this behavioural evidence with the electrophysiological evidence in this paper, I interpret the emerging pattern as follows. The principal sites of action of insect repellents are on the legs and antennae, and to a lesser extent on the labial palps. These areas possess thin walled chemoreceptors which are the only parts of insects where chemicals have access through the cuticle to the sensory tissue. The chemoreceptors on both the antennae and legs can respond to both vapours and liquids, and do so in a similar electrophysiological way. In practice insect antennae are rarely in contact with the substrate and therefore play little part in the behavioural response to contact repellency. In practice the vapour phase of repellents is more important than the liquid phase because both the antennae and legs are exposed to vapours and the antennae have more chemoreceptors than do the legs. The antennal receptors of American cockroaches respond to attractant vapour but the leg receptors do not, therefore either there are two types of receptor neuron on the antennae (one for attractants and one for repellents) or the antennal receptor neurons differ from those of the legs in being able to respond to both attractants and repellents. The latter theory would require that there are different biochemical mechanisms involved in the reception of attractants as compared with repellents and irritant chemicals. Now the response of the American cockroach antenna to banana vapour was considerably reduced after the preparation had been exposed to repellents. If the olfactory receptors for attractants are capable of responding to repellents by a separate mechanism, then this reduction in attractant response must be due to adaptation along the nerve axon and not to competition at the site of action. On the other hand, if the neurons responsible for the reception of repellents are separate from those responsible for the perception of attractants, they cannot act independently or repellents would not cause a reduction in the attractant response.

Repellent treated mosquitoes do not display their normal response to humidity and carbon dioxide (Wright, 1962). The receptors for both humidity and carbon dioxide are found on the antennae of insects and closely resemble other chemoreceptors in structure, many of them having pores opening to the surface. Some of these receptors are also sensitive to heat (Lacher, 1964).

If repellents can stimulate olfactory, gustatory, hygro- and carbon dioxide receptors in insects, and fail to stimulate the mechanoreceptors only because the concerned neurons are not exposed, then the confused behaviour of repellent treated insects noted by Khan (1965) is not surprising. I do not think that the sensory mechanisms of insects are so inelegant in function as to allow total disruption of their powers of discrimination by repellents, especially since the standard insect response to high concentration of repellent is oriented repulsion.

There is convincing electrophysiological evidence for the presence of distinct neurons which respond to general or irritant chemical stimuli (Hodgson, 1957; Boeckh, Kaissling and Schneider, 1965). These 'generalist' neurons are commonly associated in the receptor end organs with 'specialist' neurons which respond to a more restricted class of chemicals such as sugar (Hodgson, 1957), or attractants (Schneider, 1962). Even hygroreceptors contain at least two neurons and carbon dioxide sensory are similar in structure to other chemoreceptors (Lacher, 1964). The 'specialist' neurons of chemosensory organs respond to attractants or stimulants such as sugar, and the 'generalist' neurons respond to a wider range of chemical stimuli. Inhibition of the 'specialist' impulse potentials by 'generalist' receptor potentials could account for the observations that repellents reduce the response to attractants and disrupt the behavioural response to humidity and carbon dioxide. Inhibition, however, does not account for the general sensitivity of nerve tissue to irritant chemicals (Roys, 1954).

The known facts of repellency are best accounted for by combining a two neuron system with an overall sensitivity to irritant chemicals and repellents, as follows.

All chemoreceptors in insects (including hygroreceptors and carbon dioxide receptors) contain at least two types of sensory neurons, the 'generalists' which respond to a wide spectrum of chemical stimuli, and the 'specialists' which respond selectively to a narrow range of chemical stimuli at a much greater sensitivity.

Both types of receptor can respond to general irritant chemicals such as repellents, through the common chemical sense. At very low concentrations of repellent stimulus, only the 'specialist' receptors would respond, resulting in attraction.

At higher concentrations of repellent, the 'specialist' receptors for all types of attractant stimuli including water, carbon dioxide and heat are stimulated and some of the 'generalist' neurons are stimulated, causing slight decrease in activity of the 'specialist' impulse potentials. The result is confusion, disorientation and various types of abnormal behaviour. At a very high concentration of repellents, total stimulation of the 'generalist' neurons produces total decrease in activity of the 'specialist' impulse potential resulting in active repellency.

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